DECLARATION

I, Bob van Gemen, declares as follows

I am graduated from the University of Amsterdam, The Netherlands, in Molecular Biology and obtained my PhD Degree from the University of Leiden, The Netherlands, based on a thesis entitled: "The KsgA methylase of Eschericia Coli".

I am a former employee of the Company to which US patent application Serial No 09/463,352 has been assigned and had the position of Research Group Leader and Senior Research Scientist.

Presently I am Chief Executive Officer of Primagen Holding BV, a biotech Company residing at Meibergdreef 59, 1105 BA Amsterdam, The Netherlands.
Under my authority and supervision the experiments, explained in more detail in the following paragraphs of this declaration, were carried out at the laboratories of Primagen, located at the premises of the Academic Medical Centre of the University of Amsterdam. I still serve the Company, to which US patent application Serial No 09/463,352 has been assigned, as an advisor.

I am familiar with the contents of the US patent application Serial No 09/463,352, filed January 21, 2000 for Nucleic acid Sequences that can be used as primers and probes in the amplification and detection of all subtypes of HIV-1. And I have noticed the remarks of the Examiner made in his Official Action of July 31, 2001

The following report describes the experiments carried:

Background

Currently, there are several commercially available assays on the market to detect and/or quantify HIV-1 viral RNA levels in a sample. We focussed on NASBA-based assays. The current NASBA-based assay available on the market is the NucliSens HIV-1 QT/QL assay with primers located in the *gag* gene. The disadvantage is that isolates from subtype A and CRFAE_01 are under-quantified and subtype G, as well as group O and N isolates are not detected at all (1;3;5). A correlation has been found between the extent of underestimation of viral RNA levels and the amount of mismatches between primers - probes and the amplicon as reflected by the wild-type nucleotide sequence (3).

In order to improve the subtype detection and/or quantification, we identified highly conserved sequence regions in the HIV-1 LTR and leader sequence. These regions were taken to develop primer pairs that should be able to detect/quantify isolates from any HIV-1 subtype or group.

Goal:

In an end-point detection assay (based on electrochemiluminescence [ECL]) after amplification, a primer pair was found that could detect isolates from all subtypes with a sensitivity of approximately 50 copies RNA or less per reaction (3). This primer pair was taken as the reference primer set.

This (reference) LTR-primer pair [which is claimed in claim 1 of the patent application and described in publications by de Baar et al. (2-4) was compared with several other primer pairs located in the same highly conserved regions of the LTR and neighbouring "leader" region with respect to their suitability for NASBA amplification of HIV-1 RNA . An important characteristic feature of the reference primer pair is its sensitivity to less than 50 copies RNA. Suitable other primer pairs must reach this sensitivity as well and preferably be more sensitive and/or robust.

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Method:

NASBA primers located in the LTR and leader region were designed and synthesised by methods well known in the art. Purification via denaturing poly-acryl amide gels was performed by standard operating procedures. Combinations of primers were tested and compared to the performance characteristics of the said reference LTR-primer set.

Results:

All primer pairs covered by claim 1 resulted in a more sensitive assay in comparison with LTR-primer pairs not covered by the claim.

The reference primer pair was found to be the primer pair of choice on the basis of sensitivity and robustness.

Conclusions:

The results show clearly that not just any primer pair with similar design characteristics (highly conserved regions for broad detection) in the LTR and/or *gag*-leader region will automatically result in a highly sensitive assay for detection of HIV-1 RNA.

Experiments

Part I:

We investigated 5 primer pairs in the same region or close to the region that is amplified by the reference primer pair R. In figure 1, the location of these primer pairs is depicted. The primer pairs A, B, and C have a second primer [p2] which is identical to the second primer of the reference primer pair R and have a varying first primer [p1], slightly different from the first primer of the reference primer pair. The primer pairs A, B, and C as well as the reference primer pair R are covered by claim 1 of the patent application. The primer pairs D and E are not covered by claim 1 of the patent application. Primer pair D has a first primer p1 within the conserved LTR U5 region but outside the claimed sequence of claim 1 and has the same second primer [p2] as in the reference primer pair. Primer pair E has the same first primer as in the reference primer pair but a different second primer.

These primer pairs were all tested in combination with two different molecular beacons [probes] for real-time amplification-detection if applicable. The sequence of the primers is depicted in table 1. Each of the primer combinations has been tested for at least 2 times. Representative results are depicted in figures in Appendix A. As can be seen by comparing the newly tested primer sets versus the reference primer set, none of the primer sets results in more sensitive detection of HIV-1 RNA than the reference primer set. The primer pairs A, B and C are comparable with the reference primer pair; the primer pairs D and E are not as good as the reference primer set.

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Table 1. Names and sequences from the used primers.

NAME	P1/P2	SEQUENCE
P025	P1	T7-AGA GGG GCG CCA CTG CTA GAG A
P027	P2	CTC AAT AAA GCT TGC CTT GA
P189	P2	CCC TCA GAT GCT GCA TAW AAG CAG C
P200	P1	T7-GAG GGA TCT CTA GTT ACC AGA
P201	P1	T7-AGA AGC ACT CAA GGC AAG CTT TAT TGA
P202	P2	GGG TCT CTC TIG BTA GAC CAG
P203	P2	GGG TCT CTC TTG BTA GAC CAG
P216	P2	AGT AGT GTG TGC CCG TCT GT
AL-P1	P1	T7- AGA GTC GGG CGC CAC TGC TAG A
BL-P1	P1	T7- AGA GGT TCG GGC GCC ACT GCT A
DL-P1	P1	T7- AGA GCT GTT CGG GCG CCA CTG C

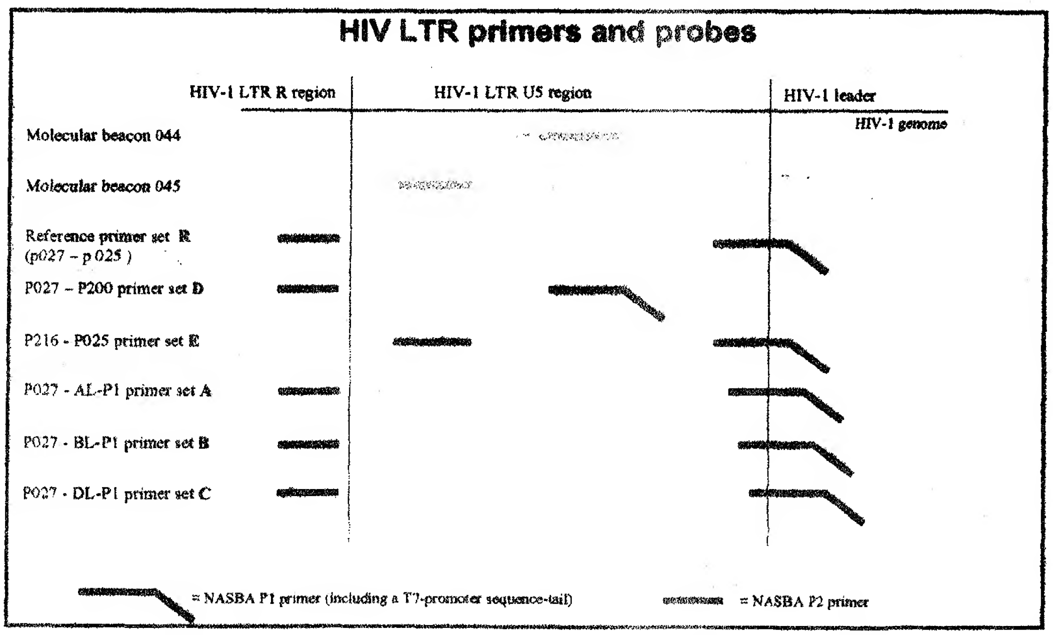

Figure 1. Schematic representation of the position of various primer pairs that were tested for their sensitivity on HIV-1 RNA (1).

Part II

Besides the U5 and leader region, also the R-region of the LTR contains highly conserved sequence stretches. Additionally, each genomic molecule of HIV contains 2 of these regions. This means that the sensitivity would increase with a factor 2 without any optimisation if this region is amplified. The primers that have been designed in the R-

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region have been depicted schematically in figure 2. From some of the primer combinations, representative results are depicted in figures in Appendix B. Those combinations that are not depicted resulted in **very** low sensitivity (often not more than 10^{5-6} copies RNA as detection limit). Not each primer combination is tested with all molecular beacons that were available, since generally the sensitivity did not improve when using another molecular beacon. It is clear from the results that none of the tested primer pairs were as good as the reference primer pair in terms of sensitivity and robustness.

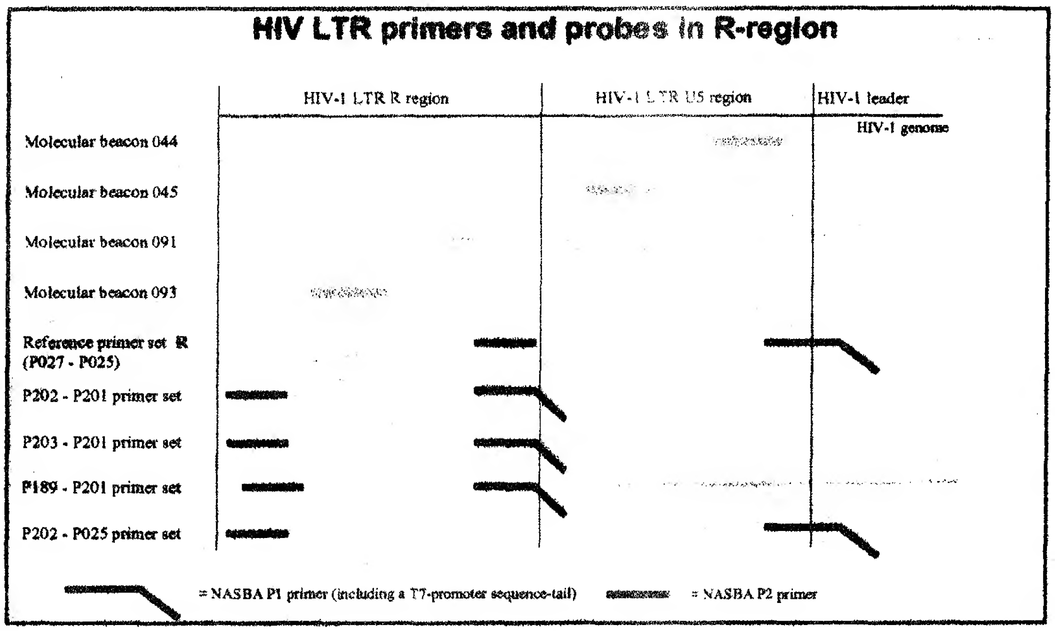

Figure 2. Schematic representation of the position of various primers that were tested for their sensitivity on HIV-1 RNA (2).

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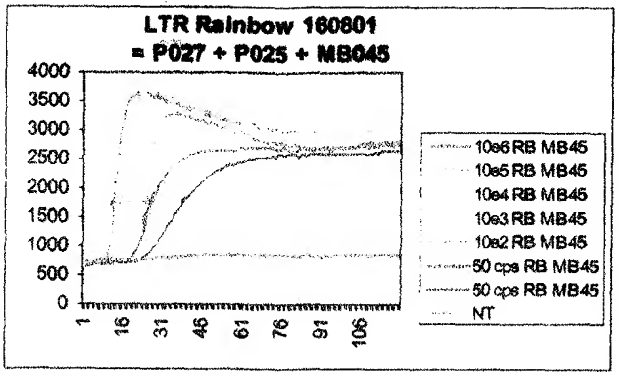
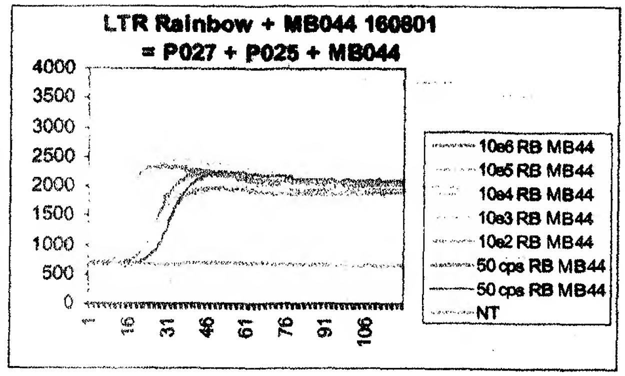
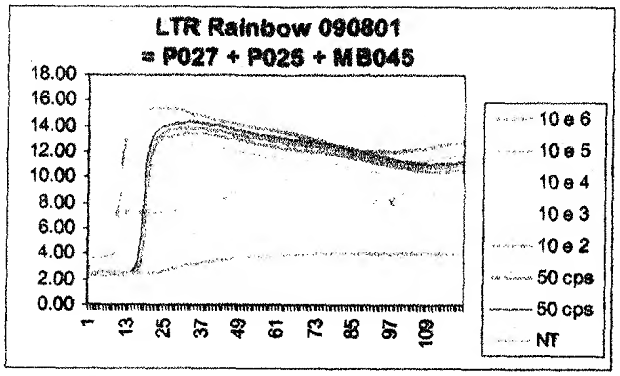
Appendix A



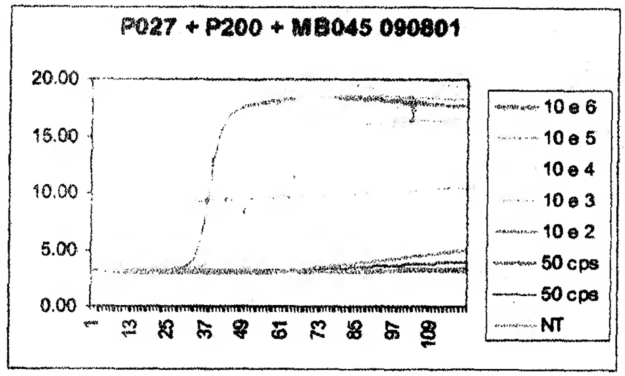
Reference primer set (+MB045 =standard)



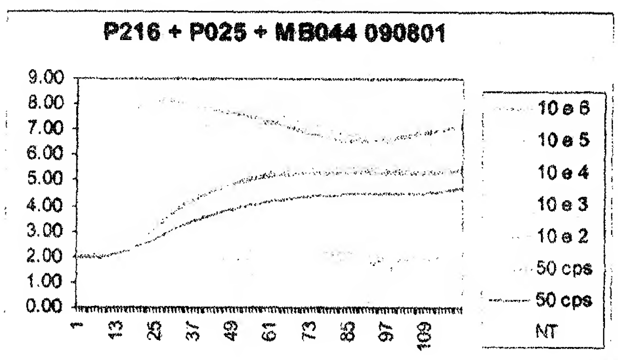
Reference primer set (+MB044)



Reference primer set (+MB045)

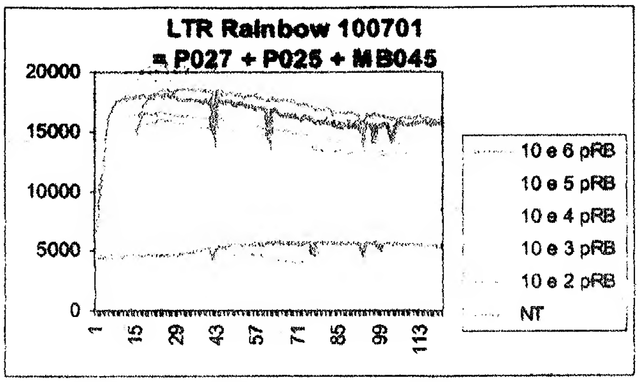


P027 + P200 + MB045

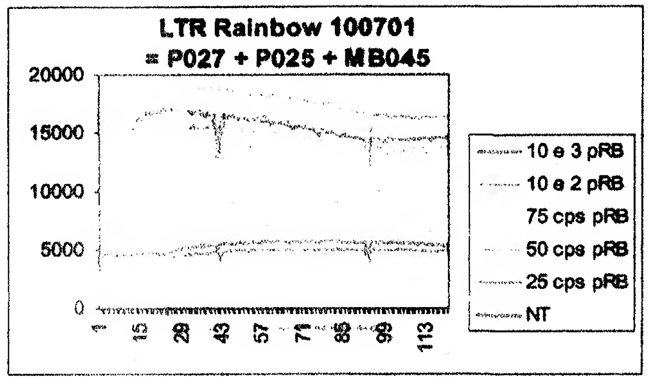


P216 + P025 + MB044

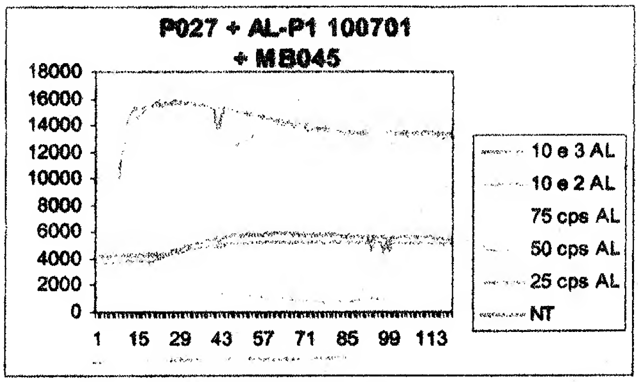
BvG



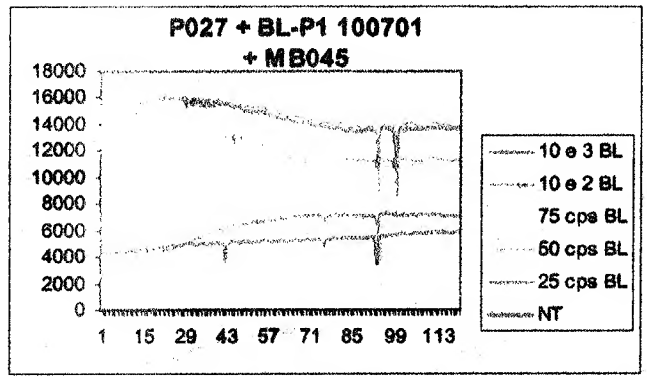
Reference primer set (+MB045)



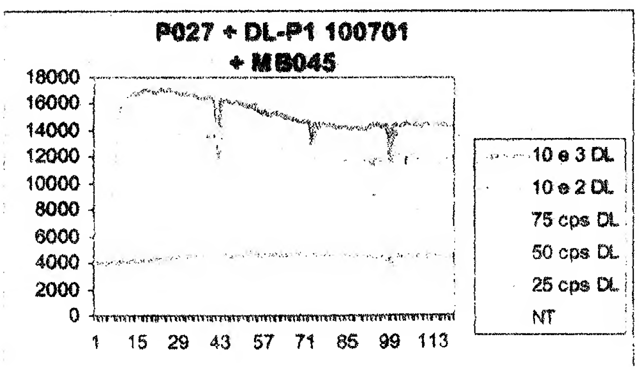
Reference primer set (+MB045)



P027 + AL-P1 + MB045



P027 +BL-P1 + MB045



P027 + DL-P1 + MB045

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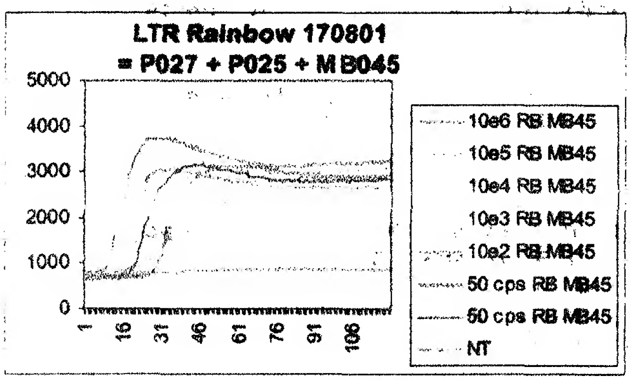
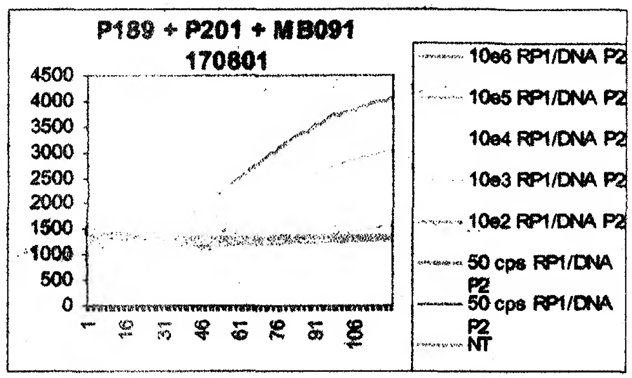
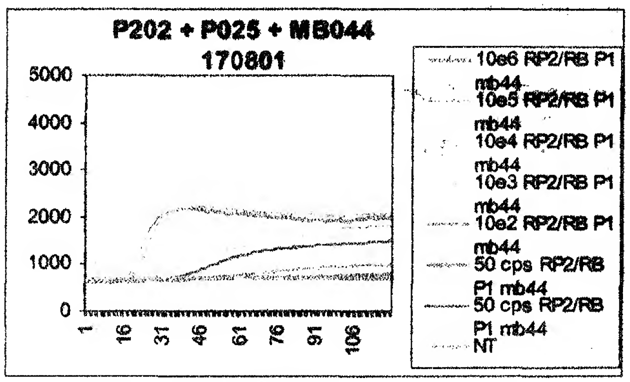
Appendix B



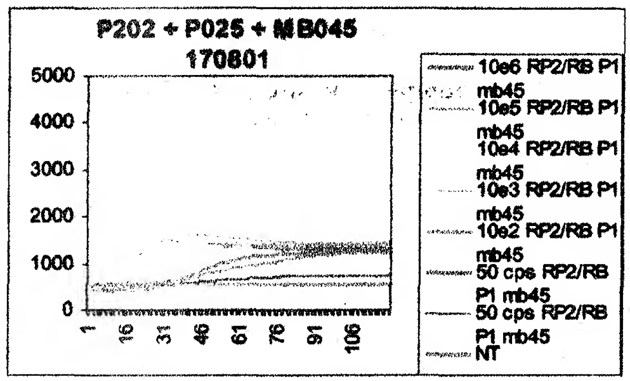
Reference primer set (+MB045)



P189 + P201 +MB091



P202 + P025 +MB044



P202 + P025 +MB045

All of the statements made above of the undersigned declarants' own knowledge are true and all statements made on information and belief are believed to be true. The undersigned acknowledges that willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and may jeopardize the validity of the above-referenced application or any patent issuing thereof.

Signed at Amsterdam:

Dr Bob van Gemen
Date: 19 March 2002

References

1. **Alaeus, A., K. Lidman, A. Sonnerborg, and J. Albert**. 1997. Subtype-specific problems with quantification of plasma HIV-1 RNA. AIDS **11**:859-865.

2. **de Baar, M. P., E. C. Timmermans, M. Bakker, E. de Rooij, B. Van Gemen, and J. Goudsmit**. 2001. One-tube real-time isothermal amplification assay to identify and distinguish human immunodeficiency virus type 1 subtypes A, B, and C and circulating recombinant forms AE and AG. J.Clin.Microbiol. **39**:1895-1902.

3. **de Baar, M. P., A. M. van der Schoot, J. Goudsmit, F. Jacobs, R. Ehren, K. H. van der Horn, P. Oudshoorn, F. De Wolf, and A. De Ronde**. 1999. Design and evaluation of a human immunodeficiency virus type 1 RNA assay using nucleic acid sequence-based amplification technology able to quantify both group M and O viruses by using the long terminal repeat as target. J.Clin.Microbiol. **37**:1813-1818.

4. **de Baar, M. P., M. W. van Dooren, E. de Rooij, M. Bakker, B. Van Gemen, J. Goudsmit, and A. De Ronde**. 2000. Single rapid real-time monitored isothermal RNA amplification assay for quantification of human immunodeficiency virus type 1 isolates from group M, N, and O. J.Clin.Microbiol. **39**:1378-1384.

5. **Parekh, B., S. Phillips, T. C. Granade, J. Bagge, D. J. Hu, and R. Respess**. 1999. Impact of HIV type 1 subtype variation on viral RNA quantitation. AIDS Res.Hum.Retroviruses **15**:133-142.

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